

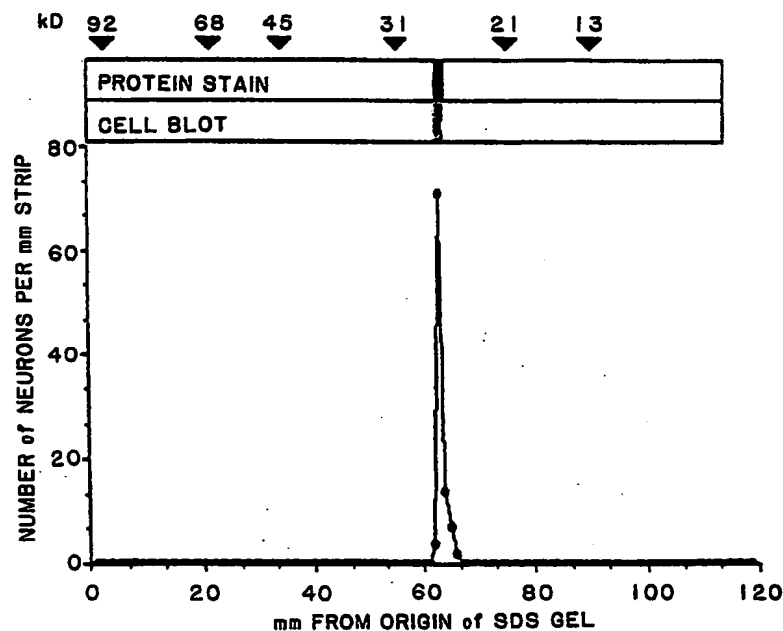
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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US90/01390 <b>(22) International Filing Date:</b> 14 March 1990 (14.03.90) <b>(30) Priority data:</b> 323,761 15 March 1989 (15.03.89) US <b>(71) Applicant:</b> THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 2199 Addison Street, Berkeley, CA 94720 (US). <b>(72) Inventors:</b> MANTHORPE, Marston ; 12418 Kestrel Street, San Diego, CA 92129 (US). VARON, Silvio ; 7502 Hillside Drive, La Jolla, CA 92037 (US). <b>(74) Agents:</b> CAMPBELL, Cathryn et al.; Pretty, Schroeder, Brueggemann & Clark, 444 South Flower Street, Suite 2000, Los Angeles, CA 90071 (US).		<b>(81) Designated States:</b> AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).  Published <i>With international search report.</i>

**(54) Title:** PURIFIED CILIARY NEUROTROPHIC FACTOR**(57) Abstract**

The present invention provides highly purified mammalian Ciliary Neurotrophic Factor (CNTF) having a specific activity greater than  $2 \times 10^7$  TU/mg protein and a method for obtaining such highly purified CNTF. In addition to having an activity greater than  $2 \times 10^7$ , highly purified CNTF is characterized by a molecular weight of about 28 kD as determined by SDS-PAGE under either reducing or non-reducing conditions; a PI of about 5.0 to about 5.4; not being inactivated by anti-NGF antibodies; not being inactivated by SDS or reducing agents; and supporting *in vitro* survival of E8 chick ciliary ganglion neurons, E12 sympathetic ganglion neurons, E10 chick dorsal ganglion neurons and neonatal mouse dorsal root ganglion neurons but not E8 chick dorsal root ganglion neurons; and being sensitive to trypsin and chymotrypsin digestion. The highly purified CNTF may be attached to a solid support, which may in turn be used, for example, to promote the survival of neurons.

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Purified ciliary neurotrophic factor.

This invention relates to growth factors and more specifically to a newly purified growth factor which supports parasympathetic motor neurons of the ciliary ganglion.

5 This invention was made with Government support under Grant No. NS16349 awarded by the NINCDS of the NIA. The Government has certain rights in this invention.

10 BACKGROUND OF THE INVENTION

Neuronotrophic factors are protein which dramatically affect the performance of nerve cells. The first neuronotrophic factor, Nerve Growth Factor (NGF), was  
15 discovered and purified almost 40 years ago. NGF is a 26 kD basic dimeric protein present in minute amounts in most, if not all tissues, and is required for the survival of developing peripheral sensory and sympathetic neurons in vitro and in vivo. It also affects cholinergic neurons in  
20 the brain that are thought to be involved in cognitive processes. The current view is that NGF is provided to the neuron in vivo by certain cells to which the neuron connects (e.g., muscle or nerve cells) and/or by neighboring glial cells (e.g., Schwann cells or astroglia)  
25 to which the neuron is apposed. NGF is thought to be required not only for the survival of the neurons but also for the maintenance and stimulation of neurite growth (axonal and dendritic processes) and the synthesis of function-related neuronal enzymes.

30 NGF acts on developing peripheral ("PNS") and central ("CNS") nervous system neurons and may thus provide useful treatments of related developmental deficits in the human fetus or child. NGF has recently been shown to work on mechanically- or age-impaired cholinergic neurons in the  
35 adult rat CNS, pointing to its potential use for i)

regeneration of damaged CNS nerve cells, ii) functional improvements of age-related memory deficits, and iii) prevention and/or reversal of nerve cell degeneration in patients with Alzheimer's, Parkinsons and related disorders.

Other neuronotrophic growth factors, or growth factors having neurotrophic activities, have also been identified, including the Fibroblast Growth Factors, and Epithelial Growth Factor. For the past ten years these studies have prompted investigators to predict that other neuronotrophic factors exist which act on neurons other than those acted on by NGF.

One specific neuron population, the chick embryo cholinergic parasympathetic motor neurons of the ciliary ganglion ("CG neurons"), is not supported by NGF. In the late 1970's, it has recognized the "CG neurons" would not survive in vitro unless the neuronal culture was supplemented with tissue extracts or with culture media preexposed to isolated muscle cells (Helfand et al., Proc. Natl. Acad. Sci. USA 80:2091-2024 (1978); Nishi and Berg, Proc. Natl. Acad. Sci. USA 74:5174-5175 (1977); Collins et al., Exp. Cell Res. 113:39-45 (1978); Varon et al., Brain Res. 173:29-45 (1979)). "Ciliary Neuronotrophic Factors" or "CNTFS" were described and preliminarily characterized which are specifically required for the survival of embryonic chick ciliary ganglion neurons but are also able to support certain NGF-responsive neurons. Chick Eye CNTF was shown to be a 204 kD protein with an isoelectric point of 5.0 which supported the survival not only of CG neurons but cultured avian and mammalian sensory neurons and avian sympathetic neurons as well. (Adler and Varon, Brain Res. 437-448 (1980); Landa et al., Dev. Biol. 74:401-408 (1980); Manthorpe et al., J. Neurochem 34:69-75 (1980); Babin et al., J. Neurochem. 43:1468-1478 (1984); Manthorpe et al., J. Neurochem. 38:225-242 (1982)).

CNTF isolated from rat sciatic nerves has been isolated and has been reported to have been "purified" (Manthorpe et al., Brain Res. 367:282-286 (1986)). This 24 kD protein was found to be distinct from chick eye CNTF.

5 However, further work on rat nerve CNTF revealed that the preparation described in Manthorpe (1986), supra, is in fact, heavily contaminated with other chemically related inactive proteins.

10 Additionally, the identification of a neurotrophic factor that promotes the survival of cholinergic parasympathetic ciliary neurons was described by Watters and Hendry, J. Neurochem. 49:705-713 (1987). SDS-PAGE of the factor showed two bands of about 22 and 23 kD.  
15 However, the SDS-PAGE procedure resulted in a large loss of biological activity and irreversible denaturation of the factor.

Growth factors, particularly those exhibiting  
20 nuerontrophic activities, have great potential utility for promoting the growth or regeneration of specific populations of cells. In order to be therapeutically useful, however, such growth factors must be highly purified and must retain their activity in the purified  
25 state. There thus exists a need for methods of purifying neurotrophic growth factors and for characterizing these purified factors. The present invention satisfies these needs and provides related advantages as well.

30

#### SUMMARY OF THE INVENTION

The present invention provides highly purified mammalian Ciliary Neurotrophic Factor (CNTF) having a specific activity greater than  $2 \times 10^7$  TU/mg protein and a  
35 method for obtaining such highly purified CNTF. In addition to having an activity greater than  $2 \times 10^7$ , highly purified CNTF is characterized by a molecular weight of

about 23 to 28 kD as determined by SDS-PAGE under either reducing or non-reducing conditions; a PI of about 5.0 to about 5.4; not being inactivated by anti-NGF antibodies; not being inactivated by SDS or reducing agents; and supporting in vitro survival of E8 chick ciliary ganglion neurons, E12 sympathetic ganglion neurons, E10 chick dorsal ganglion neurons and neonatal mouse dorsal root ganglion neurons but not E8 chick dorsal root ganglion neurons; and being sensitive to trypsin and chymotrypsin digestion. The highly purified CNTF may be attached to a solid support, which may in turn be used, for example, to promote the survival of neurons.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the quantitative survival of purified chick embryo neurons selectively on the purified 28 kilodalton CNTF band on a Western blot after SDS-PAGE.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a highly purified neuronotrophic factor termed Ciliary Neuronotrophic Factor, or CNTF. The highly purified CNTF has a specific activity of at least  $2 \times 10^7$  TU/mg protein, and retains activity after submission to SDS-PAGE and Western blotting.

Highly purified CNTF can be obtained from mammalian peripheral nerve tissue, such as rat sciatic nerve. Because it is present in such low concentration, a large amount of tissue must be used. An aqueous extract of the tissue was prepared, as by homogenation, preferably in the presence of proteolytic inhibitors. The extract was then submitted to SDS-PAGE and the band in the 25 to 30 kD region cut out and the protein extracted, as by electroelution. Those eluates containing peak CNTF activity, as determined by bioassay, were pooled.

Extraneous matter, such as residual pieces of gel, was removed from the pooled fractions, as by centrifugation and the pooled fractions submitted to reverse phase high performance liquid chromatography. Fractions exhibiting high activity were again pooled and submitted to SDS-PAGE, resulting in a band at approximately 28 kD which exclusively exhibited CNTF activity. The material obtained from the 28 kD band is highly purified CNTF.

Highly purified CNTF retains its biological activity after submission to SDS-PAGE and Western blotting. Highly purified CNTF was heated to 90°C for 10 minutes in the presence of SDS and  $\beta$ -mercaptoethanol. Highly purified CNTF was then subjected to SDS-PAGE and Western blotting, using methods well known in the art. The highly purified CNTF exhibits one sharp protein band corresponding to a molecular mass of 28 kD on analytical SDS gels and Western blots. This band possesses high biological activity after being either eluted directly from the SDS gel and provided to test neurons in soluble form or after being electrophoretically transferred onto nitrocellulose paper and being provided to test neurons in an immobilized form. Test neurons supported by this processed CNTF included embryonic chick day 8 CG, day 10 dorsal root ganglia (DRG) and 11 sympathetic ganglia (SG) and neonatal mouse DRG. The specific activity of the highly purified CNTF for E8 CG was  $2 \times 10^7$  TU/mg protein.

Various types of cultured neurons were tested to determine which were supported by highly purified CNTF. Published data on other factors from the following sources was used as a comparison:

Bovine heart Ciliary Neurotrophic Factor (bhcNTF):  
Watters and Hendry, J. Neurochem 49:705-713 (1987).

Bovine basic Fibroblast Growth Factor (FGF): Unsiker

et al., Proc. Natl. Acad. Sci. USA 84:5459-5463 (1987).

Mouse Submaxillary Nerve Growth Factor (NGF): Lindsay et al., Dev. Biol. 112:319-328 (1985).

5 Bovine brain-derived Neuronotrophic Factor (BDNF): Lindsay, et al., Supra, and Barde et al., EMBO J. 549-553 (1982).

10 Table I presents a comparison of the cells supported by these neuronotrophic factors. Values given are the percentage of neurons in the ganglion tissues whose neuronal survival is supported by the indicated factor.

15

TABLE I

	<u>Neuron Type</u>	Highly Purified	<u>bhCNTF</u>	<u>FGF</u>	<u>NGF</u>	<u>BNF</u>
		<u>CNTF</u>				
20	c CG 8	100	60	100	0	0
	c DRG 8	0	25	0	100	100
	c DRG 11-12	100	25	0	100	100
	c SG 11-12	100	25	0	100	0
	r SCGn	nd	nd	nd	100	nd
25	m DRGn	100	nd	nd	100	nd
	r NG	0	nd	nd	0	100

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nd = not reported

30 The specific activity of the highly purified CNTF is greater than  $2 \times 10^7$  TU/mg protein. In contrast, bhCNTF has been reported to have an activity of  $4.2 \times 10^4$  TU/mg protein. This highly purified CNTF is over 200 times as potent, and possibly also purer, than  
35 bhCNTF.

Moreover, indicated above, highly purified CNTF retained biological activity after being subjected to heat and SDS detergent. In contrast, bhCNTF has been reported to be completely inactivated by such treatment. Watters and Hendry, J. Neurochem. 49:705-713 (1987).

CNTF can be attached to a solid support, such as for example, to nitrocellulose paper or a plastic prosthesis. In this form the material is useful, for example, as a neural bridge.

CNTF can be attached to a solid support, such as by Western blotting to nitrocellulose paper after SDS-PAGE (Carnow et al., J. Neuroscience 5:1965-1971, 1985; Rudge et al., Dev. Brain Res. 32:103-110, 1987, which are incorporated herein by reference), as is for example, the prototype neuronotrophic factor, Nerve Growth Factor (Pettmann, et al., J. Neuroscience 8:3624-3630, 1988, which is incorporated herein by reference). In this form the material is useful as a neuronal bridge. For example, certain tissue materials, such as fetal brain tissue, adult rat sciatic nerve or placental amnion membrane have been grafted into experimentally injured adult rat brain or nerve tissue and shown to support the limited regeneration of damaged nerve cells (Kramer et al., Brain Research 210:153-172, 1981; David and Aguayo, Science 214:931-934, 1981; Davis et al., Science 236:1105-1109, 1987; Danielsen et al., Dev. Brain Res. 39:39-50, 1988, which are incorporated herein by reference). Also, uncoated nitrocellulose paper has been grafted into damaged adult rat brain and spinal cord tissue and shown to serve to a limited extent as a nerve bridge. Nitrocellulose coated with CNTF can provide bridges for the stimulation of brain regeneration.

One procedure for preparing CNTF bridges is to submit the purified CNTF to SDS-PAGE and Western blotting by standard methods. The 28 kD CNTF band region is cut out, and the remaining protein binding sites on the paper blocked with a biologically inactive protein such as purified albumin. The CNTF coated and blocked paper is inserted between the stumps of a regenerating peripheral nerve (such as has been done with Fibroblast Growth Factor, Danielsen et al., J. Neuroscience Res. in press, 1989, which is incorporated herein by reference) or within a damaged central nervous system pathway. The nitrocellulose itself has been shown to be relatively inert and does not elicit an inflammatory reaction. The immobilized CNTF can stimulate the apposing damaged nerves to regrow axons across the paper and into the denervated regions.

#### EXAMPLE I

##### 20 PROCEDURE FOR OBTAINING HIGHLY PURIFIED CNTF

1000 adult rat sciatic nerves (wet weight = 25 gm) were homogenized using a Tekmar Tissumizer (Tekmar, Co., Cincinnati, OH) at top speed in 200 ml water containing proteolytic inhibitors (0.1 mM phenyl methyl sulfonyl fluoride or PMSF (Sigma, St. Louis, MO) + 100 Units/ml kallikrein inactivator (Calbiochem, La Jolla, CA)), the homogenate was centrifuged for 1 hour at 100,000 x g and the supernate collected and stored in 20 ml aliquots at -76°C.

Each 20 ml of supernate was thawed and 5X sample buffer added to make 1X (1X = 10% glycerol, 4% beta-mercaptoethanol, 2.3% sodium dodecyl sulfate (SDS), and 0.0625 M Tris buffer, pH 6.8). This solution was then heated at 95°C for 10 minutes and cooled to 4°C, 0.004% bromophenol blue was added; this fraction is

termed "Crude Extract."

This Crude Extract was then submitted to preparative SDS-PAGE using a 180 X 120 X 6 mm thick  
5 7.5-20% polyacrylamide gradient slab gel. The gel lane (including the 20-30 kD protein band region) then cut into about 10 X 2-3mm long slices. Each slice in the appropriate region, i.e., 25 to 30 kD, was electroeluted into 1 ml of 0.1% SDS using an  
10 electroelution chamber (CBS Scientific, Del Mar, CA).

Bioassays using embryonic day 8 chick ciliary ganglion (CG) nerve cells were then performed to determine which eluates contain the peak of CNTF  
15 biological activity, described in detail in a recent review (Manthorpe, M., et al. (1989) Ciliary Neuronotrophic Factors, In "Nerve Growth Factors," R.A. Rush, ed., John Wiley and Sons, Ltd., New York, pp. 31-56, which is incorporated herein by reference).  
20 Briefly, ninety six-well Costar A/2 microplates were coated with polyornithine hydrobromide and laminin after which 25  $\mu$ l/well culture medium (Dulbecco's Modified Eagle's Medium plus 10% fetal calf serum) was added. Serial two-fold dilutions were carried out by  
25 adding 25  $\mu$ l of test sample in culture medium to wells at one end of the plate, mixing the contents, and transferring 25  $\mu$ l of the mixed contents sequentially from one well to the next. Within two hours 25  $\mu$ l/well of culture medium containing 500 freshly  
30 purified CG neurons was added. The cultures are maintained at 37°C in a 5% CO<sub>2</sub>-95% air humidified incubator for 16 hours when 5  $\mu$ l of the vital dye, MTT (Sigma Cat. # M2128; 1.5 mg/ml in culture medium) was added and the culture incubated for an additional 8  
35 hours. At the end of the 24 hour culture period, and 8 hours after the addition of MTT, the blue crystals within the remaining viable neurons were solubilized

(0.08 N hydrochloric acid in isopropanol). The intensity of the blue solution, which is proportional to the number of surviving neurons in each well, was then measured and plotted using a microplate reader interfaced with a computer. One Trophic Unit (TU)/ml is defined as that amount of CNTF activity per ml of culture medium (i.e., the final dilution) supporting half-maximal neuronal survival (i.e., optical density). Those fractions exhibiting the highest activity were pooled. Usually there were two to three peak active fractions representing 2 to 3 ml of eluate from each batch of 50 nerves.

These steps were repeated 20 times until the entire 200 ml of supernate from the 1000 nerves was processed. The active fractions were pooled to about 50 ml and 5 ml aliquots were stored at -76°C until the next step. This resulting fraction was called the "Electroeluate."

Each 5 ml pool was thawed, centrifuged for 10 minutes at 1000 X g to sediment any small residual pieces of polyacrylamide gel carried over from the electroelution step and the supernate collected. Each 5 ml of gel-free supernate was then submitted to reverse phase high performance liquid chromatography on a 250 cm X 4.6 mm C-4 300A microbore column (e.g., HiPore™ Reverse Phase Column #RP304; Bio-Rad, Inc., Cat No. 125-0550 or Vydac™ Cat No. 214TP54) using a 30 minute ramp to 45% acetonitrile followed by a 180 minute 45-60% acetonitrile gradient in 0.1% trifluoroacetic acid. Individual 0.5 ml fractions were collected into 5 µl 10% SDS to make the final eluates 0.01% SDS. Since the peak of CNTF activity elutes at about 50% acetonitrile, a 10 µl aliquot was removed from each fraction and assayed for CNTF bioactivity. The rest of each fraction was dried in

a Speedvac™ (Savant, New York, NY) by vacuum centrifugation and stored at -76°C until use. The peak of CNTF activity eluted in two to four 0.5 ml fractions and contained an average of about 0.2% of the total eluted protein and most of the loaded biological activity.

The peaks from each of the reactions from the 1000 nerve prep were thawed, resuspended in 50 µl sample buffer, pooled into a common fraction of about 1 ml. This fraction was called the "HPLC Eluate."

The HPLC eluate pool was submitted to analytical SDS-PAGE using a 15-25% polyacrylamide gradient in a low tris buffer system. The prep at this stage contains two prominent bands, one at about 30 kD and the other at about 28 kD, as well as a few minor bands in the region. CNTF biological activity as determined by two techniques (by elution from the gel and assay of the eluate and by a cell blot technique, as described below) was exclusively associated with the 28 kD band.

The CNTF was then collected free of the 30 kD and the few other minor bands by cutting 1 cm X 1 mm regions out of the appropriate region 23 to 28 kD and eluting them into 200 µl of water; the eluates were assayed for CNTF activity and the active bands pooled; the peaks were then pooled and the pool concentration by Speedvac™ (Savant, New York, NY) to about 100 µl. This fraction was called "Highly Purified CNTF."

The following Table II illustrates the various yields in the above fractions:

TABLE II

Fraction	Vol	Tu/ML	Mg/ml	TU	Mg	TU/mg
Crude Extract	200	40,000	4.00	8,000,000	800	10,000
Electroeluate	50	55,000	0.320	2,700,000	16	172,000
HPLC Eluate	1	170,000	0.033	680,000	0.125	5,440,000
Highly Purified CNTF	0.1	6,400,000	0.060	320,000	0.003	106,000,000
% Yield	-	-	-	4	0.00038	-

Fold Purification in specific activity: Crude = 1; Electroeluate = 17.2; HPLC Eluate = 544; CNTF final product = 10,600.

## EXAMPLE II

CHARACTERIZATION OF HIGHLY PURIFIED CNTF

## A. Biological Activity

The biological activity of highly purified CNTF, prepared as described in Example I, was determined to be greater than  $2 \times 10^7$  TU/mg protein. CNTF and NGF activities were determined using purified microcultures set up as described in Example I, except that either purified E8 chick ciliary or dorsal root or E11-12 chick sympathetic or neonatal mouse dorsal root ganglion neurons were used as test cells. For antibody experiments, the test neurons were presented with 50 TU/ml of the indicated trophic factor and serial dilutions of the test antibody (i.e., rabbit anti-mouse Nerve Growth Factor or rabbit anti-bovine basic or acidic Fibroblast Growth Factor) and neuronal survival quantified spectrophotometrically as in the trophic factor assays. Typical measured activities were greater than  $10^8$  TU/mg protein.

## B. Amino Acid Sequence

Amino acid sequence analysis was carried out on highly purified CNTF prepared by the procedure described in Example I. Since the amino terminus of the protein was blocked, the highly purified CNTF was digested with trypsin for sequence determination of the derived fragments. SDS-PAGE eluates from several preparations of the highly purified CNTF of Example I were pooled (final volume = 1.8 ml) and lyophilized. SDS was extracted from the residual by the acetone extraction method of Königsberg and Henderson (Königsberg, W.H. and Henderson, L., Methods Enzymol. 91:254-259 (1983)). 0.88 ml acetone, 0.05 ml triethylamine, 0.05 ml acetic acid and 0.02 ml H<sub>2</sub>O were combined and added to the residual in an eppendorf centrifuge tube. After 2 hours at -20°C, the sample was centrifuged at 15,000 rpm for 9 minutes. The supernatant was removed and 0.5 ml cold acetone was again centrifuged at 15,000 rpm for 9 minutes and the supernatant removed.

Trypsin digestion was carried out as follows. The protein pellet was dried and resuspended in 10 µl of 0.1% SDS and heated to 100°C for 3 minutes after which 100 µl trypsin buffer (2.5 mM CaCl<sub>2</sub>, 0.1 M Tris:HCl; pH 8.0) and 0.3 µg trypsin were added. The reaction was incubated at 37°C overnight after which 0.2 µg of trypsin was added and the sample incubated for an additional 4 hours at 37°C.

Tryptic fragments were separated by reverse phase HPLC (Hewlett Packard #HP109Q) using a Vydac C-18 column (2 mm x 15 cm). Fragments were eluted in a gradient of 5-65% acetonitrile in 0.1% trifluoroacetic acid. A peak eluting at 24.5 minutes (approximately 22% acetonitrile) was selected for sequence analysis

by the Edman degradation technique using an automated sequenator (Applied Biosystems model 477A). The fragment gave a single, unambiguous amino acid sequence indicating that the peak contained a single, homogenous peptide. The amino acid sequence of this peptide was as follows:

X S E M T E A E R.

On SDS-PAGE, highly purified CNTF exhibits one band having a molecular mass between about 23,000 and 28,000 da, depending on the molecular mass standard proteins used to calibrate the SDS-PAGE system. When run with a prepared mixture from Bio-Rad, Laboratories (Richmond, CA) consisting of Phosphorylase b (97,400 da), bovine serum albumin (66,200 da), ovalbumin (42,699 da), soybean trypsin inhibitor (21,500) and lysozyme (14,400), which does not contain a protein in the 22,000 to 30,000 da range, a weight of approximately 28,000 da was obtained. When rerun with other molecular weight standards which included alpha-chymotrypsinogen-A (25,200 da; Sigma Chemical Co., St. Louis, MO, Cat. No. C4879 Type II), CNTF ran just ahead of this marker and the molecular weight plots using the new markers show the CNTF to have a molecular mass as low as 23,000 da.

### EXAMPLE III

#### SUPPORT OF CCG8 NEURONS

In order to determine the biological activity of CNTF, purified as described above, the following "cell blotting" technique, as described in J. Neurosci. 5:1965-191 (1985) and Dev. Brain Res. 32:103-110 (1987) both of which are incorporated herein by reference, was used. Briefly, 200 TU of highly

purified CNTF, prepared as in Example I, were applied to SDS-PAGE. The electrophoretic lane was Western blotted to nitrocellulose and cCG8 neurons cultured on the blotted lane. Initially, neurons attached  
5 uniformly along the entire lane, but after 48 hours in vitro neurons only survived on the 28 kD CNTF band. Figure 1 shows the quantitative survival of cCG8 neurons selectively on the 28 kD CNTF band.

10 In contrast, bhCNTF is reported to exhibit two bands, at 22 and 23 kDs, but neither of these bands exhibited activity towards cCG8 neurons. Watters and Hendry, supra.

15 Although the invention has been described with reference to the presently-preferred embodiment, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only  
20 by the following claims.

## WE CLAIM:

1. Highly purified biologically active CNTF having the following characteristics:

- a. has a specific activity greater than  $2 \times 10^7$  TU/mg protein;
- b. molecular weight of about 28 kD as determined by SDS-PAGE under either reducing or non-reducing conditions;
- c. PI of about 5.0 to about 5.4;
- d. is not inactivated by anti-NGF antibodies;
- e. is not inactivated by SDS or reducing agents;
- f. supports in vitro survival of E8 chick ciliary ganglion neurons, E12 sympathetic ganglion neurons, E10 chick dorsal ganglion neurons and neonatal mouse dorsal root ganglion neurons, but not E10 chick dorsal root ganglion neurons; and
- g. is sensitive to trypsin and chymotrypsin digestion.

2. A method of purifying biologically active CNTF, comprising the steps of:

- a. preparing a aqueous extract of adult rat sciatic nerve;
- b. submitting the extract to SDS gel electrophoresis;
- c. electroeluting a peak CNTF activity;
- d. fractionating the electroeluate by C-4 reverse phase hydrophobic HPLC;
- e. eluting of CNTF activity in the 50% acetonitrile region;
- f. submitting of the most active HPLC fractions to SDS gel electrophoresis; and
- g. eluting the peak of CNTF activity.

3. Highly purified, biologically active CNTF substantially equivalent to that produced by the method of claim 2.

4. Highly purified CNTF attached to a solid support.

5. A method of promoting the survival of neurons by providing highly purified CNTF attached to a solid support.

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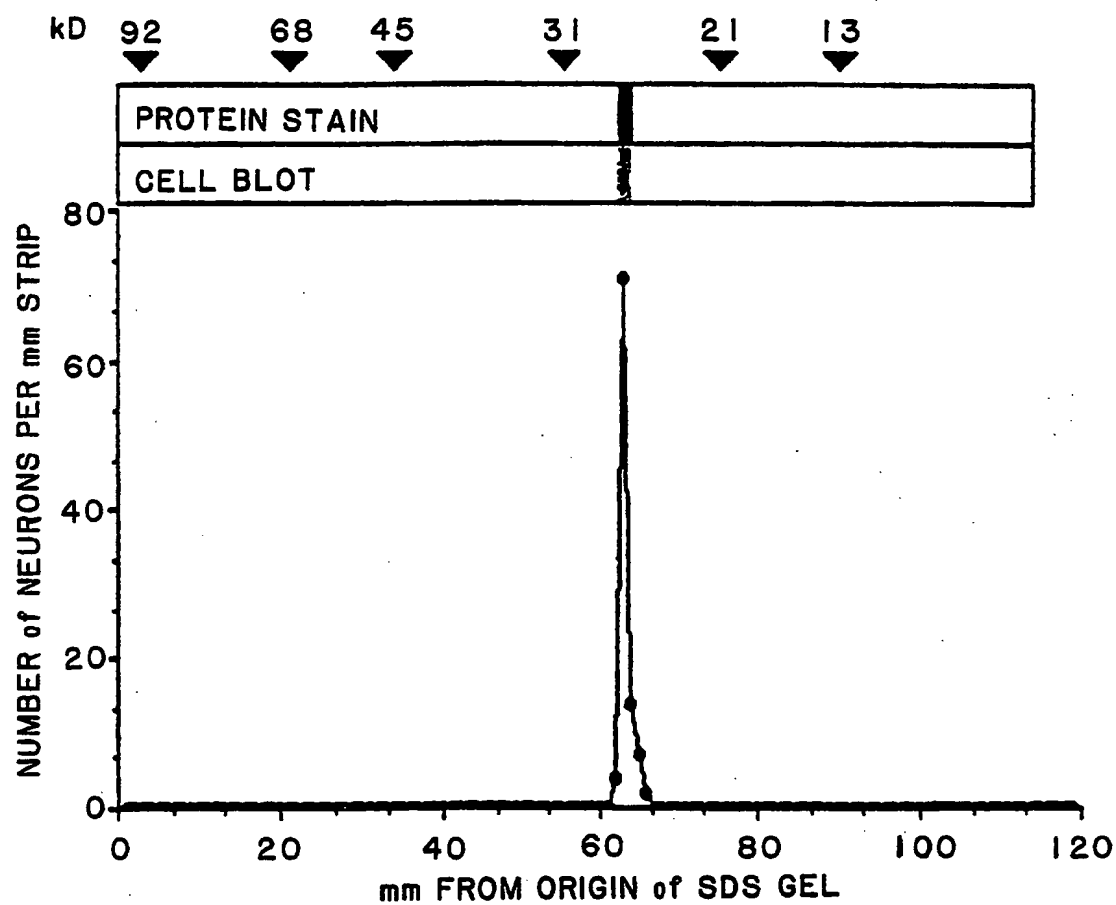


FIG. 1

SUBSTITUTE SHEET

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/01390

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup> According to International Patent Classification (IPC) or to both National Classification and IPC IPC5: C 07 K 13/00, C 07 K 3/28, C 07 K 17/00, A 61 K 37/02																	
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched<sup>7</sup></div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 20%; border: 1px solid black; text-align: left;">Classification System</th> <th style="border: 1px solid black; text-align: left;">Classification Symbols</th> </tr> <tr> <td style="border: 1px solid black; height: 40px; vertical-align: bottom;">IPC5</td> <td style="border: 1px solid black; vertical-align: bottom;">C 07 K; A 61 K</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the extent that such Documents are included in Fields Searched<sup>8</sup></div>			Classification System	Classification Symbols	IPC5	C 07 K; A 61 K											
Classification System	Classification Symbols																
IPC5	C 07 K; A 61 K																
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b> <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%; text-align: left;">Category *</th> <th style="width: 60%; text-align: left;">Citation of Document,<sup>11</sup> with indication, where appropriate, of the relevant passages<sup>12</sup></th> <th style="width: 30%; text-align: left;">Relevant to Claim No.<sup>13</sup></th> </tr> </thead> <tbody> <tr> <td style="vertical-align: top;">X    Y A</td> <td style="vertical-align: top;">JOURNAL OF NEUROCHEMISTRY, Vol. 49, No. 3, 1987, Diane J. Watters et al: "Purification of a Ciliary Neurotrophic Factor from Bovine Heart", see page 705 - page 713 page 705, the abstract, page 706, column 2, page 707-708, column 2, the part "preparative SDS-PAGE"</td> <td style="vertical-align: top;">1,3    4,5 2</td> </tr> <tr> <td style="text-align: center;">--</td> <td></td> <td></td> </tr> <tr> <td style="vertical-align: top;">X    Y A</td> <td style="vertical-align: top;">RESEARCH ARTICLES, Vol. 246, 1989, Leu-Fen H. Lin et al: "Purification, Cloning, and Expression of Ciliary Neurotrophic Factor (CNTF)", see page 1023 - page 1025 page 1023, column 1, second part, page 1025, note 5</td> <td style="vertical-align: top;">1,3    4,5 2</td> </tr> <tr> <td style="text-align: center;">--</td> <td></td> <td></td> </tr> </tbody> </table>			Category *	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>	X    Y A	JOURNAL OF NEUROCHEMISTRY, Vol. 49, No. 3, 1987, Diane J. Watters et al: "Purification of a Ciliary Neurotrophic Factor from Bovine Heart", see page 705 - page 713 page 705, the abstract, page 706, column 2, page 707-708, column 2, the part "preparative SDS-PAGE"	1,3    4,5 2	--			X    Y A	RESEARCH ARTICLES, Vol. 246, 1989, Leu-Fen H. Lin et al: "Purification, Cloning, and Expression of Ciliary Neurotrophic Factor (CNTF)", see page 1023 - page 1025 page 1023, column 1, second part, page 1025, note 5	1,3    4,5 2	--		
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>																	
<b>IV. CERTIFICATION</b> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border: 1px solid black; padding: 5px;">           Date of the Actual Completion of the International Search             13th June 1990         </td> <td style="width: 50%; border: 1px solid black; padding: 5px;">           Date of Mailing of this International Search Report             03.07.90         </td> </tr> <tr> <td style="border: 1px solid black; padding: 5px;">           International Searching Authority             EUROPEAN PATENT OFFICE         </td> <td style="border: 1px solid black; padding: 5px;">           Signature of Authorized Officer              MISS T. TAZELAAR         </td> </tr> </table>			Date of the Actual Completion of the International Search  13th June 1990	Date of Mailing of this International Search Report  03.07.90	International Searching Authority  EUROPEAN PATENT OFFICE	Signature of Authorized Officer  MISS T. TAZELAAR											
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International Searching Authority  EUROPEAN PATENT OFFICE	Signature of Authorized Officer  MISS T. TAZELAAR																

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	BRAIN RESEARCH, Vol. 367, 1986, Marston Manthorpe et al: "Purification of Adult Rat Sciatic Nerve Ciliary Neuronotrophic Factor ", see page 282 - page 286 page 284, column 1, lines 1-4 and table 1, page 283, column 1, the parts extract preparation and fractionation procedure	1,3
Y		
A	--	4,5 2
A	Dialog Information Services, File 55, Biosis 81-90, BIOSIS number 79033551, Barbin G et al: "Purification of the chick eye ciliary neuronotrophic factor" J Neurochem 43 (5), 1984, 1468-1478	1-5
Y	Dialog Information Services, File 55, Biosis 81-90, BIOSIS number 87029258, Pettmann B et al: "Biological activities of nerve growth factor bound to nitrocellulose paper by western blotting", J Neurosci 8 (10), 1988, 3624-3632	4,5
A	WO, A1, 9000568 (THE UNIVERSITY OF TENNESSEE RESEARCH CORPORATION) 25 January 1990, see claim 2	2
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**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO. PCT/US 90/01390**

**SA 35542**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the European Patent Office EDP file on **07/05/90**  
The European Patent office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9000568	25/01/90	NONE	

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